RECOMBINANT CALF-CHYMOSIN AND A PROCESS FOR PRODUCING THE SAME

This invention relates to recombinant calf-chymosin and a process for producing the same.

TECHNICAL FIELD OF THE INVENTION:

Chymosin is an enzyme which is particularly useful in the preparation of cheese. Natural sources of chymosin include stomachs of calf, goat, lamb, porcine and the like. However, commercial chymosin is primarily obtained from the fourth stomach of mild fed calves. Alternate sources of chymosin have been developed particularly because of the decrease in calf production. Production and extraction of commercially valuable proteins from recombinant microorganisms encouraged the study of producing and purifying microbially produced chymosin. However, a process suitable for commercial scale production and recovery of chymosin has not been developed so far.

OBJECTS OF THIS INVENTION:

The main objective of this invention is to provide an efficient process for expressing prochymosin gene and its conversion into enzymatically active pure chymosin.

SUMMARY OF THE INVENTION:

Calf-chymosin gene is isolated preferably from the fourth stomach of milk fed calf tissues. Recombinant calf-chymosin is produced by cloning chymosin gene with bacterial expression vector PET21b and is transformed to E-coli strain. This E-coli strain containing recombinant calf-chymosin gene is fermented under suitable conditions preferably in a culture medium developed by us. This medium contains the following

Peptone – 12g/l

Yeast Extract – 24g/l

Sodium chloride – 10g/l

Prochymosin produced during fermentation is subjected to denaturation by increasing the pH of the medium to 10-11. The suspension then diluted and the pH reduced to about 8 for effective renaturation of the protein. The prochymosin thus obtained is then acidified for activation and is further processed.

This invention relates to a process for producing recombinant calf-chymosin which comprises the steps of isolating calf-chymosin gene, cloning the same in bacterial expression vector PET 21b, transforming said cloned vector into cells of E-coli, fermenting said E-coli strains to produce pro-chymosin, converting said prochymosin to chymosin and subsequently recovering the recombinant calf chymosin.

This invention also includes recombinant calf-chymosin having the following amino acid sequence:

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    MetAlaSerIle ThrArgIle ProLeuTyr LysGlyLysSer LeuArgLys AlaLeuLys
  1 ATGGCTAGCA TCACTAGGAT CCCTCTGTAC AAAGGCAAGT CTCTGAGGAA GGCGCTGAAG
    TACCGATCGT AGTGATCCTA GGGAGACATG TTTCCGTTCA GAGACTCCTT CCGCGACTTC
    GluHisGlyLeu LeuGluAsp PheLeuGln LysGlnGlnTyr GlyIleSer SerLysTyr
 61 GAGCATGGGC TTCTGGAGGA CTTCCTGCAG AAACAGCAGT ATGGCATCAG CAGCAAGTAC
    CTCGTACCCG AAGACCTCCT GAAGGACGTC TTTGTCGTCA TACCGTAGTC GTCGTTCATG
    SerGlyPheGly GluValAla SerValPro LeuThrAsnTyr LeuAspSer GlnTyrPhe
121 TCCGGCTTCG GGGAGGTGGC CAGCGTGCCC CTGACCAACT ACCTGGATAG TCAGTACTTT
    AGGCCGAAGC CCCTCCACCG GTCGCACGGG GACTGGTTGA TGGACCTATC AGTCATGAAA
    GlyLysIleTyr LeuGlyThr ProProGln GluPheThrVal LeuPheAsp ThrGlySer
181 GGGAAGATCT ACCTCGGGAC CCCGCCCCAG GAGTTCACCG TGCTGTTTGA CACTGGCTCC
    CCCTTCTAGA TGGAGCCCTG GGGCGGGGTC CTCAAGTGGC ACGACAAACT GTGACCGAGG
    SerAspPheTrp ValProSer IleTyrCys LysSerAsnAla CysLysAsn HisGlnArg
241 TCTGACTTCT GGGTACCCTC TATCTACTGC AAGAGCAATG CCTGCAAAAA CCACCAGCGC
    AGACTGAAGA CCCATGGGAG ATAGATGACG TTCTCGTTAC GGACGTTTTT GGTGGTCGCG
    PheAspProArg LysSerSer ThrPheGln AsnLeuGlyLys ProLeuSer IleHisTyr
301 TTCGACCCGA GAAAGTCGTC CACCTTCCAG AACCTGGGCA AGCCCCTGTC TATCCACTAC
    AAGCTGGGCT CTTTCAGCAG GTGGAAGGTC TTGGACCCGT TCGGGGACAG ATAGGTGATG
    GlyThrGlyLys MetGlnGly IleLeuGly TyrAspThrVal ThrValSer AsnIleVal
361 GGGACAGGCA AGATGCAGGG GATCCTGGGC TATGACACCG TCACTGTCTC CAACATTGTG
    CCCTGTCCGT TCTACGTCCC CTAGGACCCG ATACTGTGGC AGTGACAGAG GTTGTAACAC
    AspIleGlnGln ThrValVal LeuSerThr GlnGluProGly AspValPhe ThrTyrAla
421 GACATCCAGC AGACAGTAGT CCTGAGCACC CAGGAGCCCG GGGACGTCTT CACCTATGCC
    CTGTAGGTCG TCTGTCATCA GGACTCGTGG GTCCTCGGGC CCCTGCAGAA GTGGATACGG
    GluPheAspGly IleLeuGly MetAlaTyr ProSerLeuAla SerGluVal LeuAspThr
481 GAATTCGACG GGATCCTGGG GATGGCGTAC CCCTCGCTGG CCTCAGAAGT ACTCGATACC
    CTTAAGCTGC CCTAGGACCC CTACCGCATG GGGAGCGACC GGAGTCTTCA TGAGCTATGG
    GlyPheAspAsn MetMetAsn ArgHisLeu ValAlaGlnAsp ValPheSer ValTyrMet
541 GGCTTTGACA ACATGATGAA CAGGCACCTG GTGGCCCAAG ACGTGTTCTC GGTTTACATG
    CCGAAACTGT TGTACTACTT GTCCGTGGAC CACCGGGTTC TGCACAAGAG CCAAATGTAC
    AspArgAsnGly GlnGlyAsn MetPheThr LeuGlyAlaIle AspProSer TyrTyrThr
601 GACAGGAATG GGCAGGGAAA CATGTTTACC CTGGGGGCCA TCGACCCGTC CTACTACACA
    CTGTCCTTAC CCGTCCCTTT GTACAAATGG GACCCCCGGT AGCTGGGCAG GATGATGTGT
    GlySerLeuHis TrpValPro ValThrVal GlnGlnTyrTrp GlnPheThr ValAspSer
661 GGGTCCCTGC ACTGGGTGCC CGTGACAGTG CAGCAGTACT GGCAGTTCAC TGTGGACAGT
    CCCAGGGACG TGACCCACGG GCACTGTCAC GTCGTCATGA CCGTCAAGTG ACACCTGTCA
    ValThrIleSer GlyValVal ValAlaCys GluGlyGlyCys GlnAlaIle LeuAspThr
721 GTCACCATCA GCGGTGTGGT TGTGGCCTGT GAGGGTGGCT GTCAGGCCAT CCTGGACACG
    CAGTGGTAGT CGCCACACCA ACACCGGACA CTCCCACCGA CAGTCCGGTA GGACCTGTGC
    GlyThrSerLys LeuValGly ProSerSer AspIleLeuAsn IleGlnGln AlaIleGly
781 GGCACCTCCA AGCTGGTCGG GCCCAGCAGC GACATCCTCA ACATCCAGCA GGCCATTGGA
    CCGTGGAGGT TCGACCAGCC CGGGTCGTCG CTGTAGGAGT TGTAGGTCGT CCGGTAACCT
    AlaThrGlnAsn GlnTyrAsp GluPheAsp IleAspCysAsp AsnLeuSer TyrMetPro
841 GCCACACAGA ACCAGTACGA TGAGTTTGAC ATCGACTGCG ACAACCTGAG CTACATGCCC
    CGGTGTGTCT TGGTCATGCT ACTCAAACTG TAGCTGACGC TGTTGGACTC GATGTACGGG
    ThrValValPhe GluIleAsn GlyLysMet TyrProLeuThr ProSerAla TyrThrSer
901 ACTGTGGTCT TTGAGATCAA TGGCAAAATG TACCCACTGA CCCCCTCCGC CTATACCAGC
    TGACACCAGA AACTCTAGTT ACCGTTTTAC ATGGGTGACT GGGGGAGGCG GATATGGTCG
    GlnAspGlnGly PheCysThr SerGlyPhe GlnSerGluAsn HisSerGln LysTrpIle
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 961 CAGGACCAGG GCTTCTGTAC CAGTGGCTTC CAGAGTGAAA ATCATTCCCA GAAATGGATC
     GTCCTGGTCC CGAAGACATG GTCACCGAAG GTCTCACTTT TAGTAAGGGT CTTTACCTAG
     LeuGlyAspVal PheIleArg GluTyrTyr SerValPheAsp ArgAlaAsn AsnLeuVal
1021 CTGGGGGATG TTTTCATCCG AGAGTATTAC AGCGTCTTTG ACAGGGCCAA CAACCTCGTG
     GACCCCCTAC AAAAGTAGGC TCTCATAATG TCGCAGAAAC TGTCCCGGTT GTTGGAGCAC
     GlyLeuAlaLys AlaIle***
1081 GGGCTGGCCA AAGCCATCTG A
     CCCGACCGGT TTCGGTAGAC T
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In the above sequence, amino acids shown in red indicate sequence variation of chymosin gene of our invention compared to the reported and published sequence.

DESCRIPTION OF THE PREFERRED EMBODIMENTS OF THE INVENTION:

Isolation of calf-chymosin gene:

Total RNA was isolated from the 4th stomach of calf tissue. The tissue was frozen and ground to a fine powder. The powder was transferred to a 50ml centrifuge tube containing 10ml of denaturing buffer (4M Guanidine thiocyanate, 25mM Sodium citrate pH 7.0, 0.5% Sarkosyl and 0.1M 2-Mercaptoethanol). To this 1ml of 2M Sodium acetate pH 4.0, 10ml of Phenol and 2ml of Chloroform were added and kept on ice for 20 min. Later, it was cenrifuged for 15 min. at 12,000rpm, 4°C. The upper aqueous phase was transferred carefully into a new tube to which an equal volume of isopropanol was added and kept at –20°C for 1 hr. The RNA was precipitated at 15,000rpm for 15min., at 4°C. The RNA pellet was washed with 70% ethanol and dissolved in 1ml of denaturing buffer followed by two successive extractions with phenol : chloroform : isoamyl alcohol (30 : 29 :1). The RNA was precipitated with 1/10 volume of 3M sodium acetate (pH 5.2) and 2.5 volume of ethanol and dissolved in 500µl of DEPC treated water.

For the synthesis of first strand of the cDNA, 10µg of RNA was dissolved in 16µl DEPC water and the following components were added: 2µl of 10mM dNTP mix, 2µlof 1µg of reverse primer (5'-TGT GGG GAC AGT GAG GTT CTT GGT C-3), 4µl of 10X RT buffer (200mM Tris-HCl, pH 8.4, 500mM KCl), 8µl of 25mM $MgCl_2$, 4µl of 0.1M DTT, 2µl of Rnase out Recombinant Rnase inhibitor, 2µl (50 units) of Superscript II reverse transcriptase. The mixture was incubated for 50min at 42°C and the reaction is stopped by inactivating the enzyme at 70°C for 15 min.

PCR amplification of prepro chymosin was performed using the 50ng of 1st strand cDNA with a reverse primer (5'-TGT GGG GAC AGT GAG GTT CTT GGT C-3'), and a forward primer (5'-ATG AGG TGT CTC GTG GTG CTA CTT-3') in a thermal cycler programmed as (step 1: 95-5'; step 2: 94-30sec.; step 3: 54-30sec; step 4: 72-1min; step 5: go to step 2 34 times; step 6: 72-7min; step 7: end). The PCT reaction when analyzed on 1.0% agarose gel showed an amplified band of 1.2kb. The 1.2kb fragment was cut with a sterile blade and the gel slice was dissolved in 500µl of Tris saturated phenol was added and left in liquid nitrogen for a few min. The microcentrifuge tube was allowed to come to room temperature and centrifuged for 5min at 12,000rpm, 4°C. The upper aqueous phase was extracted with phenol : chloroform : isoamyl alcohol (25 : 24 : 1) and DNA was precipitated with 1/10th volume sodium acetate and 2.5 volume ethanol at –70°C for 1 h. DNA was precipitated at 15,000rpm for 15 min. The pellet was dried and dissolved in sterile distilled water. This eluted 1.2kb fragment was ligated at SmaI site of pBSSK+ plasmid, which was then transformed in to TOP10 cells of E.coli. The recombinant clones were selected (blue white screening) and checked with restriction digestion analysis of the plasmids. Recombinant plasmid was taken as a template and a PCR was performed using a forward primer (5'-GAT ATA CAT ATG GCT AGC ATC ACT AGG ATC CCT CTG TAC-3') and reverse primer (5'-GCA GTA AGC TTG ACA GTG AGG TTC CTT GGT CAG CG-3') containing Nde 1 and Hind III sites. An amplified band of 1098bp was observed when the PCR product was analyzed on 1.0% agarose gel. This amplified fragment of 1098bp was eluted from the gel and ligated in pET21b expression vector at Nde 1 and Hind III sites and transformed in to BL21 cells of E.coli for the expression of the chymosin gene.

Fermentation of recombinant E.coli expressing calf chymosin:

Fermentation of E.coli cells containing recombinant calf chymosin gene was carried out in 15 L fermentor with 6 L of working volume. Fermentation was carried out in SBL Medium as herein after described using 4% inoculum as seed. The fermentation process lasts for 22-24 h., and the whole procedure can be divided into four stages:

Stage 1	:	Preparation of SBL media.
Stage 2	:	Preparation of Accessories for fermentation.
Stage 3	:	Preparation of Seed.
Stage 4	:	Process of Fermentation.

Composition of the SBL medium:

Peptone	:	12g/L
Yeast Extract	:	24g/L
Sodium Chloride	:	10g/L

These ingredients were calculated for 6 L, weighed and dissolved in 5.75 L of distilled water and later volume was made upto 6L with water, pH was set to 7.0 using 4N NaOH. 240 ml was separated out from 6L and was autoclaved separately at 15 ib/sq.inch for 20 min. The remaining 5.76 L was taken in fermentor vessel, 1ml of sigma A concentrate antifoam was added and autoclaved with the vessel at 15 ib/sq.inch for 45 min.

Preparation of supplements for fermentation:

1. Lacose:

0.4 % (w/v) lactose for 6L medium was prepared by dissolving 24g in 150ml distilled water, autoclaved at 15 ib/sq.inch for 20 min. 0.2% (w/v) lactose for 6L medium was prepared by dissolving 12g in 150ml distilled water, autoclaved at 15 ib/sq.inch for 20 min.

2. Glycerol:

0.3% (v/v) Glycerol for 6 L medium was made by adding 18ml of glycerol to 82 ml of water, autoclaved at 15 ib/sq.inch for 20 min.

3. Ampicillin:

100 mg/ml ampicillin was made by dissolving 1200mg of sodium salt of ampicillin in 12ml of distilled water. The solution was filter sterilized by passing through 0.2 microns millipore syringe filter.

4. IPTG (Isopropyl-β-D-thiogalactopyranoside):

2mM IPTG (for 6L medium) was prepared by dissolving 2.85g of IPTG in 20ml distilled water. The solution was filter sterilized by passing through 0.2 microns millipore syringe filter.

5. 4N NaOH:

100 ml of 4N NaOH was made by dissolving 16g of NaOH pellets in 90ml of autoclaved water, and after dissolution, volume is made upto 100ml with water.

Preparation of seed:

4% SBL medium (240 ml of 6L medium) was inoculated with 100µl glycerol stock of E.coli cells in 500ml bevelled flask containing 100µg/ml ampicillin. The flask was kept shaking at 37°C 250 RPM for 18 h. Optical Density (OD) of the culture was read at 600nm in Shimadzu Spectrophotometer.

Fermentation process:

The whole process of fermentation begins with inoculation of seed at 4% into SBL medium. The pre-grown seed is inoculated into 5.76 L autoclaved SBL medium. Along with the seed –0.3% glycerol, 6mL of 100µg/mL ampicillin were also added through the inlet pump. Prior to addition of seed, the fermentor was made ready by calibrating different probes like pH probe, DO probe and Temperature probe.

a) pH probe calibration:

pH probe was calibrated using standard pH 4.0 and pH 7.0 solutions.

b) Dissolved Oxygen (DO) probe calibration:

DO probe was calibrated by using 5% sodium nitrite solution for 0% DO.

c) Temperature probe calibration:

Temperature probe was checked using water at different temperatures in a standard water bath.

Fermentation conditions:

The fermentation parameters set were given as below, and the fermentation was started by quick addition of the seed into the inoculation port.

Temperature	:	37°C
pH	:	7.0
Agitation	:	350 RPM
Dissolved Oxygen	:	30 %
SLPM	:	1.25
VVM	:	0.2

After 2h. of inoculation 6ml of 100µg/ml ampicillin was added. When OD (at 600nm) reaches about 4-5 (after 3h.) 0.4 % Lactose was added. Then the temperature was reduced to 32°C. When OD reaches 7-8 (after 5h.) 0.2 % Lactose was added. Agitation speed was then increased to 450 RPM. When the OD (at 600nm) reaches 10.0, the culture batch was induced with 2mM IPTG.

pH monitoring during the fermentation process:

pH was monitored carefully during the process of fermentation from the seed inoculation stage till the end. Initially during the growth phase of bacteria the pH of the culture drops, and the pH is maintained at 7.0 using 4N NaOH. After substantial growth of bacteria, pH shoots above 7.0 and addition of 4N NaOH was completely stopped. Samples of 1ml were collected from the fermentor at different time points, viz., uninduced (immediately before IPTG addition), 3h., 6h., and 9h. after IPTG addition, and were processed for loading onto the gel for SDS-PAGE analysis.

Cell harvesting:

After running the fermentor for 20 to 24 h. following seed inoculation, and when the OD (at 600 nm) of the fermentor sample reads to ~ 20 O.D/ml, the fermentor batch was terminated by switching off all the controls.

Pelleting and storage of the cells:

After termination of the batch, the cell culture was pelleted by centrifuging at 8000x g for 10 min. Supernatant was discarded and the pellet was stored in –70°C freezer until further use.

Extraction and purification of chymosin enzyme:

E.coli cells after fermentation were suspended in 3.5 to 4 volumes of 10mM EDTA (pre-chilled, 4°C) and the suspension was incubated at 4°C for 30 to 60 min. to obtain homogeneous suspension. Later the suspension was subjected to lysis by adding equal volume of alkali solution (0.2 N Sodium hydroxide) to a final concentration of 0.1 N with continuous stirring for 15-20 min. at 4°C. For complete denaturation and effective renaturation in the subsequent step, the lysed suspension was diluted to 9-12 folds with pre-chilled (4°C) aqueous solution (H_2O). Diluted suspension was allowed to stand at 4°C for 30 min. and the pH was readjusted to 8.0 by addition of 1.0 M glycine solution to a final concentration of 56 mM and allowed to stand at 4°C for 30 min. The inactive form-pro-chymosin at this stage was incubated for 72 h. at RT (28 + 2°C) for proper refolding.

During this incubation period, the cell debris and other solid masses (Nucleic acid complexes) settles to the bottom and the supernatant can be decanted to obtain clear folded pro-chymosin.

In the subsequent steps the pH of pro-chymosin was adjusted to 2.0 for the activation.

The adjustment is mainly by addition of a buffer with pH 1.5 (1.0M Hydrochloric acid and 1.0M glycine in 0.8:1.0 ratio). The extract was kept at low pH for a period of 6-8 h. Following the above activation, the crude low pH extract was subjected to a step where by precipitated impurities can be separated. This separation can be achieved by conventional industrial separation methods such as filtration (Whatmann No.3). Hence, the process is economically efficient, can be easily scaled up for commercial production.

The supernatant or filtrate resulting from the above separation containing the extracted milk clotting enzyme can be processed in any one of the three methods. In method I the enzyme was concentrated by subjecting to sodium chloride precipitation to about 5.8-6.0 M. Precipitation was usually carried at 4°C by gradual addition of the required amount of salt and subjecting to continuous stirring for an hour after complete addition of the salt. The solution was then subjection to vaccum filtration using 0.2 nylon membranes and the supernatant free of any enzyme can be discarded. The wet precipitate was resuspended in a 4.0 pH buffer and subsequently increased to pH 5.0 and formulated for stability.

In Method II, the pH of the filtrate was increased to the pH (to 4.7; or to 5.0) and the same was maintained at 32oC for about an hour and subjected to another filtration to obtain clear chymosin. However, the higher pH ranges around 5.0 are not preferred, which may reduce the stability of the enzyme during storage might be due to the exposure of the active site during the process. Hence, the preferred method of activation is the acidification followed by precipitation.

In Method III, after acidification step the pH of the filtrate was increased to 4.7 and subjected to sodium chloride precipitation. The precipitate was dissolved as mentioned in Method I.

The chymosin produced in all the above three methods is substantially pure, needs to be formulated to the desired specifications for final use. The salt concentration for formulation (NaCl) was brought to about 10% and a preservative such as Sodium benzoate was added. The enzymatic strengths were measured in terms of IMCU (international milk clothing units). In the present investigation, 6000-9000 IMCU per gram of biomass was obtained.

Solutions and reagents required for chymosin process:

Solution A	:	0.01M EDTA
Solution B	:	0.2N Sodium hydroxide
Solution C	:	Autoclaved water
Solution D	:	1.0M Glycine
Solution E	:	1) Solution D

II) 1.0N Hydrochloric acid

For Solution E	:	Mix (I) and (II) in 1:0.8 ratio and pH should be 1.5
Solution F	:	5.0M Sodium chloride
Solution G	:	I) Solution D

II) 0.5N Sodium hydroxide

For Solution G	:	Mix (I) and (II) in 1:0.4 ratio and should be pH 9.5
Reagent H	:	Sodium benzoate
Reagent I	:	Sodium chloride
Solution J	:	0.2M Glycine with 0.001M EDTA
Sol. J	:	Adjust the pH of solution J to 4.0 with Solution E
Sol. J	:	Adjust the pH of solution J to 5.0 with Solution E.
Reagent K	:	Trehalose-filter sterilized solution (10%).